served (236, 324). Whether biological differences in the parasite, including differences in drug sensitivity and virulence, are responsible for the treatment failure is unknown. However, high rates of genetic polymorphisms in a limited geographic region are well established (11, 22, 118, 119, 193) and may be partially responsible for the observed therapeutic failure. Alternatively, genetic differences in immune response and drug metabolism may influence the efficacy of antiamoebic drugs (72).

Various nonimidazole drugs, including nitazoxanide, paromomycin, and niridazole, reportedly have the potential to be used for treatment of microaerophilic protozoan parasite infections (62, 73). Nitazoxanide, a nitrothiazoyl-salicylamide derivative, could be used as the first-line agent against amoebiasis and other intestinal parasitic diseases in the future (246). Nitazoxanide has broad-spectrum antiparasitic activity, including activity against the protozoans *E. histolytica*, *G. intestinalis*, *T. vaginalis*, *Cryptosporidium parvum*, and *Isospora belli* and the helminths *Ascaris lumbricoides*, *Ancylostoma duodenale*, *Trichuris trichiura*, *Taenia saginata*, *Hymenolepsis nana*, and *Fasciola hepatica* (267). The mode of action for nitazoxanide is unproven, but it is predicted to inhibit PFOR. Clinical efficacy was also demonstrated in a randomized, double-blind, placebo-controlled study (267).

The disadvantages of the current antiamoebic drugs, besides their relative ineffectiveness against luminal cysts, include various side effects. Adverse effects of metronidazole include anorexia, nausea, vomiting, diarrhea, abdominal discomfort, disulfiram-like alcohol intolerance, and hypersensitivity (62, 122). Neurological side effects include dizziness, vertigo, paresthesias, and, rarely, encephalopathy or convulsions which warrant discontinuation of the drug (246). Neutrocytopenia is also associated with metronidazole. Metronidazole is also known to be mutagenic in bacteria and carcinogenic in rodents, making teratogenicity a concern (28, 44, 47, 51, 265). Metronidazole is also known to cross the placental barrier. The Food and Drug Administration (FDA) classified metronidazole as a class B risk factor for pregnancy. Accordingly, use of metronidazole for amoebiasis in pregnant women is not currently recommended (58, 59), although a causal connection between metronidazole exposure during pregnancy and birth defects has not been established (45, 63, 94). Emetine and dehydroemetine hydrochloride have serious side effects, including nausea, vomiting, cardiotoxicity, local pain, and tenderness (162). These drugs are poorly excreted into the gut and urine and accumulate at high concentrations in the liver, heart, and other tissues. Side effects of nitazoxanide include diarrhea, nausea, vomiting, abdominal pain, and flatulence (246).

### Current Chemotherapeutics for *G. intestinalis* Infection

The most commonly used drugs against giardiasis, i.e., metronidazole (250 mg three times a day for 5 to 10 days), tinidazole (100 mg three times a day for 7 days), furazolidone (100 mg four times a day for 7 to 10 days), and quinacrine (100 mg three times a day for 5 to 7 days), show up to 90% efficacy (furazolidone, <80%), which is followed by albendazole (400 mg four times a day for 5 days) and paromomycin (500 mg three times a day for 5 to 10 days) (1, 99, 125, 129). Metronidazole is considered the drug of choice, with tinidazole being an alternative (31, 99). However, metronidazole does not have an FDA indication for this use in the United States, whereas tinidazole is not available in the United States (246). A single-high-dose (2-g) regimen and/or multiple low doses (250 mg three times a day) for 5- to 7-day regimens of metronidazole were previously recommended (31). Tinidazole or ornidazole is generally recommended as a single-high-dose (2-g) regimen for treatment of giardiasis (99). Nitazoxanide received an FDA indication for the treatment of giardiasis and is available as a liquid formulation. Although reports are limited, nitazoxanide appears to be as effective as metronidazole and tinidazole (1, 6). Considering its excellent oral availability and that the major metabolites in serum (tizoxanide and glucuronide tizoxanide) exceed the 90% inhibitory concentration ($IC_{90}$) against in vitro cultures of *E. histolytica*, *G. intestinalis*, and *T. vaginalis*, nitazoxanide could be used for cases that are refractory to metronidazole treatment in the future. Alternative drugs during pregnancy include paromomycin. Other drugs, including furazolidone, quinacrine, and albendazole, should be reserved for the treatment of cases refractory to the first-line drugs. Furazolidone was often recommended for children because of its availability as a suspension (31, 149). Quinacrine and furazolidone, similar to metronidazole, are not tolerated with alcohol due to a disulfiram-like reaction (99, 149). Five of six cases refractory to metronidazole treatment were successfully treated with a combination regimen of quinacrine and metronidazole (227).

Albendazole, currently used as an anthelminthic, was first successfully used against giardiasis (340, 341) with other benzimidazoles (fenbendazole and mebendazole), with variable success. The first large-scale human study of albendazole, conducted in Bangladesh, showed a lower average efficacy than for metronidazole (120), suggesting that single high doses of metronidazole or tinidazole are advantageous with regard to patient compliance. A recent study demonstrated that combinations of albendazole and phenyl-carbamate derivatives were more effective against albendazole-resistant *G. intestinalis* strains (150). Nitazoxanide was also effective for treatment of giardiasis cases resistant to metronidazole and albendazole therapy (1). The side effects of benzimidazoles are similar to those of metronidazole (anorexia, vomiting, and metal taste) but of lower intensity (155).

### Current Chemotherapeutics for *T. vaginalis* Infection

Drugs commonly used against *T. vaginalis* infection are generally similar to those described above for *E. histolytica* or *G. intestinalis*. Metronidazole is administered orally in one of the following regimens: 250 mg three times a day for 7 days, 500 mg twice a day for 7 days, or a single 2-g dose. The last regimen is usually favored because of better compliance and because a lower total dose is required for successful treatment. Metronidazole can also be administered intravenously at a dose of 500 mg to 2 g over 20 min (62). Reported cure rates for oral and intravenous regimens were similar (85 to 95%) and increased in cases where the sexual partners were treated simultaneously (186). There has been a reluctance to utilize nitroimidazoles for trichomoniasis in pregnant women, particularly in the United States, due to some concerns over severe side effects and the teratogenecity of metronidazole, as described above

(58, 59, 62, 279, 289, 290). It is now considered that the risk to the fetus from maternal trichomoniasis is far greater than any risk related to metronidazole exposure from the mother.

Although metronidazole is still the drug of choice (153), drug-resistant cases of trichomoniasis are considerably more common than in infections with *G. intestinalis* or *E. histolytica*. The second drug of choice for treatment of trichomoniasis is tinidazole. A recent study using >100 clinical isolates demonstrated that they showed slightly higher sensitivity to tinidazole (minimum lethal concentration [MLC], 1.0 ± 1.3 mM) than to metronidazole (MLC, 2.6 ± 1.9 mM) under aerobic conditions. However, there was no significant difference in MLCs between these nitroimidazoles under anaerobic conditions (61). The efficacies of a number of nitroimidazole derivatives other than metronidazole and tinidazole for the treatment of *T. vaginalis* infection have been investigated. Although the modes of action of these derivatives are similar, the pharmacokinetics, tissue distributions, trichomonicidal activities, and toxicities are variable for these compounds (62). Ornidazole and secnidazole are similar to tinidazole in that they have longer half-lives but lower rates of efficacy to cure infection than metronidazole. In contrast, nimorazole, a nitrothiazole derivative, is converted into two major metabolites that possess significantly higher trichomonicidal activity than metronidazole (177). Nimorazole was active against both metronidazole-sensitive and -resistant *T. vaginalis* strains (334, 335).

Intravaginal application of paromomycin was successfully used to treat recurrent trichomoniasis. However, severe side effects, including pain and mucosal ulceration, made it an unlikely candidate for clinical therapy (249). Nitazoxanide was as active against *T. vaginalis* as metronidazole (6, 48), similar to the case for infections caused by *E. histolytica* and *G. intestinalis* as described above and for *C. parvum* and *Blastocystis* (6, 100). Hamycin, an aromatic polyene related to amphotericin B, was shown to induce cell death in *T. vaginalis* and other eukaryotes by binding to ergosterols in the plasma membrane and forming pores. Although lower concentrations of hamycin effectively killed both metronizole-sensitive and -resistant *T. vaginalis* strains, toxicity both in vitro and in vivo is likely to make hamycin inapplicable for clinical use (190). Sodium nitrite, sodium nitroprusside, and Roussinn's black salt, traditionally used to prevent food contamination, exhibited trichomonicidal activity against both sensitive and resistant *T. vaginalis* strains (268). Sulfimidazole, which has two functional groups, sulfonamide and 5-nitroimidazole, exhibited activity comparable to that of metronidazole against metronidazole-sensitive strains (MLC, 10 μg/ml) and was more effective against the metronidazole-resistant strains (MLC, 40 to 60 μg/ml) (73, 196).

## DRUG TARGETS AND MECHANISMS OF RESISTANCE

### Mechanism of Action of 5-Nitroimidazole and Benzimidazoles

Metronidazole (α-hydroxyethyl-2-methyl-5-nitroimidazole), a synthetic 5-nitroimidazole (56), enters the cell and its organelles via passive diffusion. Metronidazole is relatively inert until its 5-nitro group is reduced within the cell or organelle by an appropriate electron donor such as ferredoxin. Importantly, metronidazole activation occurs only under strong reducing conditions. As oxygen is an efficient electron acceptor, increased levels of oxygen result in the impaired reduction and activation of metronidazole which could theoretically result in metronidazole resistance. Thus, sensitivity to metronidazole is influenced by oxygen tension. Metronidazole is usually activated through the acceptance of electrons from ferredoxin or flavodoxin that is reduced by PFOR (178, 218–220, 264, 308). Among microorganisms, there is a strong correlation between the presence of PFOR activity and metronidazole sensitivity (138, 218, 219, 266, 275, 308). In humans, where PFOR is absent, pyruvate dehydrogenase catalyzes the oxidative decarboxylation to form acetyl-CoA and NADH in mitochondria (see above). It was directly demonstrated by using purified PFOR and ferredoxin from *G. intestinalis* trophozoites that these Fe-S cluster-containing proteins activate metronidazole in vitro (306–308, 314). The reduction of metronidazole results in the nitro-radical form, which binds transiently to DNA to disrupt or break the nucleotide strands, leading to cell death (65, 76, 77, 182). Studies with *Escherichia coli* showed that the damage caused by the direct binding of activated metronidazole to DNA is repaired by the excision repair pathway, which was not activated in *T. vaginalis* trophozoites exposed to metronidazole (172). DNA damage may be the basis for the carcinogenicity of metronidazole in animals, although carcinogenicity of metronidazole in humans has not been demonstrated.

The cellular compartments where metronidazole is activated differ among the "amitochondriate" protists. It was proposed that metronidazole is activated in *E. histolytica* by ferredoxin in the cytosol (256). As described above, ferredoxin nitroreductase, a fusion protein of 2[4Fe-4S] ferredoxin and nitroreductase, may be a target of metronidazole in *E. histolytica* and *G. intestinalis* (229). *G. intestinalis* also has, in addition to ferredoxin-nitroreductases, an oxygen-insensitive nitroreductase that lacks the ferredoxin domain and which is also present in archaea and bacteria (229). This nitroreductase reduces and activates the nitro groups of metronidazole and furazolidone in *Helicobacter pylori* and *E. coli*, respectively. Bacterial mutants lacking this nitroreductase activity were resistant to a corresponding drug (115, 163, 327). Although a physiological electron acceptor from reduced ferredoxin in *E. histolytica* has not been unequivocally demonstrated, oxygen competes with metronidazole for electrons from the physiological electron donor. Therefore, it is conceivable that the metronidazole sensitivity of *E. histolytica* is influenced by oxygen tension, as demonstrated for *Trichomonas* (see below). In *G. intestinalis*, the terminal oxidase (NADH oxidase), which converts oxygen directly to water to scavenge for oxygen and protect the anaerobic PFOR and ferredoxins (37), seems to be a physiological acceptor of electrons from ferredoxin in the cytosol. In contrast, in *T. vaginalis*, metronidazole is metabolized and activated in the hydrogenosomes. Metronidazole competes with the terminal enzyme hydrogenase for electrons from ferredoxin (218).

The underlying mode of action of benzimidazoles, including albendazole, fenbendazole, and mebendazole, has been extensively studied in the parasitic nematode *Haemonchus contortus* (171, 187, 188, 226). Benzimidine binds to the β-tubulin monomer prior to dimerization with α-tubulin to block microtubule formation (171). More specifically, benzimidazoles bind to the high-affinity binding site on the β-tubulin monomer (187). The effect of benzimidazoles on in vitro assembly of

microtubules was investigated in benzimidazole binding assays using recombinant α- or β-tubulin and dimeric tubulin (αβ-tubulin) from *G. intestinalis*, *Encephalitozoon intestinalis*, and *C. parvum* (192).

### Drug Resistance in *E. histolytica*

A number of factors are associated with resistance against metronidazole and related 5-nitroimidazoles in "amitochondriate" protists, including a decreased uptake of metronidazole, an altered pyruvate-oxidizing metabolic pathway (168), and high oxygen tension (82). There are no reports of high levels of resistance to metronidazole in clinical isolates of *E. histolytica*, but resistant cases of *Trichomonas* and *Giardia* infections are frequently observed (see below). However, inadequate short-term exposure to metronidazole and exposure to sublethal levels of metronidazole could induce increased drug resistance (312, 314, 323). Indeed, under experimental conditions, a stepwise increase in drug concentration induced metronidazole resistance in two axenic lines of *E. histolytica* (274). Two independent laboratory strains, HM-I:IMSS and HTH-56:MUTM, developed resistance against metronidazole and grew in the presence of 10 μM metronidazole, which is normally lethal to parasites in vitro (101, 274, 323). A metronidazole-resistant line that grew in the presence of 40 μM metronidazole was developed independently (323). In the former study, the expression of SOD increased three- to fivefold in the metronidazole-resistant line (274). Unlike in *Giardia* and *Trichomonas* (see below), PFOR activity did not decrease significantly in this metronidazole-resistant line (274). In the latter study, SOD mRNA and enzyme activity (323) were increased fivefold in the resistant strain, while PFOR mRNA decreased only marginally, supporting the previous finding. Peroxiredoxin (thiol-specific antioxidant) mRNA and enzyme activity also increased by three- to fourfold, while NADPH:flavin reductase mRNA and its activity decreased by 40%. In addition, ferredoxin 1, but not ferredoxin 2, was selectively decreased in this resistant strain (323), which may reflect the specificity of metronidazole. However, further biochemical differentiation of the two ferredoxins has not been done. *E. histolytica* possesses an unusual pathway for detoxification of superoxide radicals and hydrogen peroxide, using SOD, rubrerythrin (185), NADPH:flavin oxidoreductase (38), and peroxiredoxin (323) to protect oxygen-sensitive PFOR and ferredoxin. Thus, it is conceivable that *E. histolytica* may possess a mechanism of metronidazole resistance different from those of the other two "amitochondriate" protists.

An emetine-resistant *E. histolytica* strain was developed in vitro (21, 276, 277). This emetine-resistant *E. histolytica* line overexpressed P-glycoprotein and showed some features of the multidrug-resistant phenotype (21). The accumulation of emetine by the mutant amoebae was 50% lower than in wild-type amoebae (21). However, the rate of drug entry and efflux from the parasite per se was not examined in that study. Emetine resistance was reversed by the calcium channel blocker verapamil (21, 276). It was hypothesized that *E. histolytica* actively expelled hydrophobic drugs, including emetine, by P-glycoprotein as described for multidrug-resistant tumor cells (97), because the emetine-resistant *E. histolytica* line was cross-resistant to other hydrophobic drugs such as iodoquinol and diloxanide but not to nonpolar drugs such as chloroquine and metronidazole (277). Also, the resistant strain released radiolabeled emetine more rapidly than the susceptible strain, suggesting that P-glycoprotein overexpression was responsible for emetine resistance in *E. histolytica* (277).

### Drug Resistance in *G. intestinalis*

The prevalence of clinical metronidazole-resistant cases of giardiasis is reported to be up to 20% (30, 95), with recurrence rates of up to 90% (339). Resistant organisms have been isolated from patients and characterized in various laboratories (1, 6, 7, 95, 150, 191, 310, 312, 314). Cross-resistance to tinidazole has also been demonstrated in metronidazole-resistant strains (310, 312, 314). Furanidazole-resistant *G. intestinalis* strains produced in vitro adapted more readily to quinacrine (311). In vitro development of albendazole resistance was also reported (180, 309). Albendazole resistance developed more easily in a furazolidone-resistant strain, giving rise to a multidrug-resistant phenotype (309).

PFOR expression was down-regulated fivefold in a metronidazole-resistant *G. intestinalis* line (308), which is consistent with the premise that PFOR is the primary target of metronidazole and that a decreased amount of the target is the mode of resistance in *G. intestinalis*. Recently, antisense inhibition of PFOR caused metronidazole resistance in *G. intestinalis* (64). In contrast to the case for metronidazole-resistant lines, PFOR did not change in a furazolidone- and quinacrine-cross-resistant *G. intestinalis* line (314). The activity of the next electron acceptor in the transport chain, ferredoxin I, decreased by sevenfold, while the amount of ferredoxin I decreased by only twofold (181, 314), suggesting that another layer of regulation influenced the acceptor activity of ferredoxin. In an independent study, a furazolidone-resistant line showed increased activity of NADH oxidase, which activates the drug to its free radical state (35, 37). That study concluded that furazolidone resistance is due to the reduced expression of ferredoxin and increased NADH oxidase activity, which differs from metronidazole resistance. In contrast, albendazole-resistant *G. intestinalis* strains revealed changes in the cytoskeleton, especially for β-tubulin (309), suggesting that qualitative changes in β-tubulin lead to decreased sensitivity to albendazole (191). In that study, benzimidazole analogues showed higher affinity for monomeric β- and heterodimeric αβ-tubulin derived from benzimidazole-sensitive parasites than for those from benzimidazole-insensitive organisms (191).

### Drug Resistance in *Trichomonas*

It was proposed that metronidazole resistance be categorized into "aerobic" and "anaerobic" mechanisms (62). In "aerobic resistance," ferredoxin and other components of the antioxidative system seem to play a major role. In "anaerobic resistance," reduction of hydrogenosome functions in general and of PFOR and hydrogenase activities in particular are apparently responsible for resistance. Most clinical metronidazole-resistant strains show biochemical alterations, which is consistent with the "aerobic resistance" phenotype. It is important to note that aerobic resistance can develop in vivo with therapeutic levels of metronidazole and does not require ex-

posure to incremental doses of metronidazole during prolonged treatment, as shown for the in vitro development of anaerobic metronidazole resistance by a stepwise increase in drug concentrations. There are a number of reports of clinical cases of resistance against metronidazole or 5-nitroimidazole derivatives (61, 225, 319, 336).

Because oxygen is an efficient electron acceptor, increased levels of cellular oxygen in hydrogenosomes result in the impaired reduction and activation of metronidazole. Oxygen concentrations in resistant strains were much higher than those in susceptible strains (82). The high oxygen concentrations likely inhibit accumulation of the drug, because oxygen competes with metronidazole for electrons from reduced ferredoxin. If metronidazole is not reduced, there is no concentration gradient of the drug across the plasma membrane to allow extracellular metronidazole to enter the cell. In addition, the reduced nitro free radical is oxidized back to the original compound by oxygen and in turn produces a superoxide anion (62, 245). This process is known as futile cycling and results in only limited damage to the cells via superoxide anions in comparison to cell death due to reactive nitro radicals. While "aerobic resistance" phenotypes were frequently observed in clinical isolates, anaerobic resistance is found mostly in in vitro-induced metronidazole-resistant lines. Metronidazole-resistant *T. vaginalis* strains artificially developed by increasing the drug concentration in vitro showed either reduced or absent PFOR and hydrogenase activities (169). Unfortunately, the current view of metabolic mechanisms giving rise to metronidazole-resistant trichomoniasis, both in clinical settings and in vitro, is not completely elucidated by the "aerobic" versus "anaerobic" models.

Clinical drug-resistant *T. vaginalis* isolates show various biochemical changes, e.g., decreased expression of PFOR, ferredoxin, and hydrogenase activities (62, 73, 168, 253); oxygen resistance (80, 336); and decreased oxidase activity (336). In addition, highly resistant *T. vaginalis* clinical isolates possess neither detectable PFOR activity nor PFOR and ferredoxin mRNAs (34, 73, 169, 175, 253). Earlier work suggested that hydrogenosomes could be lost in drug-resistant parasites. However, it was reported later that the organelle remained, but in a modified form (168). Although structural changes of hydrogenosomes were reported to occur in a metronidazole-resistant *T. vaginalis* strain, it is unclear whether these are primary events or are secondary to the advent of resistance (153). Also, metronidazole-resistant strains simultaneously lost multiple hydrogenosomal proteins, including ferredoxin, PFOR, malic enzyme, and hydrogenase, due to inactivation of hydrogenosomes (140, 254). The metronidazole-resistant *T. vaginalis* trophozoites showed enhanced lactate fermentation as they lost PFOR activity, PFOR mRNA, and ferredoxin mRNA and thus the pyruvate-oxidizing pathway in the hydrogenosomes (34, 73). Hrdy et al. (140) demonstrated that metronidazole is activated by electrons from ferredoxin that originate not from PFOR but from malate in this metronidazole-resistant strain. These data support the notion that trichomonads acquire high-level metronidazole resistance only after both pyruvate- and malate-dependent pathways of metronidazole activation are eliminated from hydrogenosomes. These lines of evidence also suggested that "aerobic" and "anaerobic" resistance mechanisms are not mutually exclusive.

One previous study provided contradictory evidence that there was no significant change in PFOR activity, anaerobic fermentation, and intracellular accumulation of metronidazole between metronidazole-resistant and -susceptible isolates (220). However, accumulation of [$^{14}$C]metronidazole was more inhibited under aerobic conditions in resistant isolates than in susceptible strains. Thus, they concluded that the production of electrons was not hampered but that the activation of metronidazole was reduced in the resistant isolates (220). This observation was also consistent with the premise that the metronidazole-resistant strain had reduced electron transport ability, and this therefore was classified as "aerobic resistance."

One line of evidence suggests that ferredoxin plays a major role in metronidazole resistance. The major ferredoxin purified from *T. vaginalis* trophozoites was indeed reduced by PFOR in vitro, as detected by electron paramagnetic resonance spectroscopy (116). This ferredoxin could interact with metronidazole and accept electrons from PFOR (116). Changes in the upstream transcriptional regulatory regions (nucleotide −239 upstream of the transcription initiation site) of the ferredoxin gene were also demonstrated in metronidazole-resistant strains (253). The mRNA and protein levels of ferredoxin decreased by ~50% in these strains. A recent study showed that ferredoxin gene replacement in *T. vaginalis* did not lead to in vitro metronidazole resistance (176). Thus, it remains unclear to what extent ferredoxins are involved in metronidazole resistance. The *T. vaginalis* genome project indicates that the genome is 160 to 180 Mb in size and highly repetitive. These data indicate that knockout of a single gene may not be deleterious for the parasite and that an alternative ferredoxin or flavodoxin might also be responsible for metronidazole activation in *T. vaginalis*.

Cross-resistance between different nitroimidazoles was also reported (208, 225). For instance, metronidazole-resistant clinical isolates showed partial cross-resistance to tinidazole (61, 319). However, metronidazole-resistant isolates do not always coexist with cross-resistance against other nitroimidazole compounds (61). Metronidazole-resistant isolates described by Narcisi and Secor were sensitive to a nonnitroimidazole nitrofuran, furazolidone (225), which is consistent with the notion that the mechanism of metronidazole resistance in *T. vaginalis* differs from that in *G. intestinalis* (314). Further studies need to be conducted to clarify molecular mechanisms of cross-resistance.

## SULFUR-CONTAINING-AMINO-ACID METABOLISM AS A NOVEL DRUG TARGET

### Metabolic Pathways in Protozoan Parasites under Investigation To Explore as Targets for Drug Development

Unique metabolic pathways that are present in pathogens but absent or divergent in their hosts are always potential rational targets for drug development. Among a number of pathways, some of which are listed here, the unique pathways in parasitic protists include fatty acid, isoprenoid, phospholipid, sterol, and heme biosynthesis in *Plasmodia*, *Toxoplasma gondii*, *Trypanosoma*, and *Leishmania* (32, 40, 60, 266, 291, 329); polyamine metabolism in *Trypanosoma* (259); aspartic

acid proteases (plasmepsins) and cysteine proteases (falcipains) from *Plasmodium* (52); trypanothione metabolism in *Trypanosoma* (93); thioredoxin metabolism in *Plasmodium* and *T. vaginalis* (221); protein kinases from *Plasmodium*, *Toxoplasma*, and *Eimeria* (68); the hexose transporter from *Plasmodium* (151); and the dihydroorotate dehydrogenase (23) and the mitochondrial cyanide-insensitive terminal oxidase from *Trypanosoma brucei* (159).

The number of targets in "amitochondriate" parasites currently under investigation is not sufficient considering the rapid emergence of drug resistance described above. Potential targets for the development of antiamoebic drugs include the glycolytic pathway (namely, pyrophosphate-dependent phosphofructokinase and pyruvate kinase) (103, 200), alcohol dehydrogenase 2 (90, 337), cysteine proteases (157, 252), isoprenyltransferases (170, 195), and sulfur-containing-amino-acid metabolism (233). For the development of new chemotherapeutics against giardiasis, several candidates have been investigated, including guanine phosphoribosyltransferase, a key enzyme in the purine salvage pathway (222), and biosynthesis of a novel β-(1,3)-*N*-acetyl-D-galactosamine homopolymer [including 4′-epimerase and β-(1,3)-*N*-acetyl-D-galactosamine transferase] (102, 148, 285). Encystation and excystation are unique cellular processes that fulfill the criteria for rational drug targets. In particular, the biosyntheses of mannoproteins, chitin, and β-1,3-glucan are suggested as targets for antifungal drugs against *Entamoeba* and *Giardia* (148). Possible targets of *T. vaginalis* that have been investigated include a thioredoxin-linked peroxiredoxin antioxidant system (54, 221) and sulfur-containing-amino-acid metabolism (53, 326) (see below).

Among the handful of possible targets, we propose sulfur-containing-amino-acid metabolism as one of the rational and promising pathways for the development of new chemotherapeutic agents against "amitochondriate" parasites, particularly those causing amoebiasis and trichomoniasis. First, sulfur-containing-amino-acid metabolism plays a pivotal role in virtually all organisms (233). Namely, methionine and cysteine are building blocks of proteins; *S*-adenosylmethionine is the precursor for polyamine biosynthesis and the essential methyl donor for many methyl transfer reactions, including the DNA methylation involved in the regulation of gene expression; and cysteine is a precursor for the biosynthesis of glutathione, which is an essential antioxidant. Second, there are remarkable qualitative, not quantitative, differences between the parasites and their hosts. In most of the drug targets described below, suitable targets are selectively present only in "amitochondriate" parasites and are absent in their hosts. Finally, sulfur-containing-amino-acid metabolism has also been viewed as a reasonable target for the development of drugs against infection caused by other parasitic protists, including *Plasmodium* and *Trypanosoma*, and their physiological and biological significance has been well studied (233).

## Physiological Importance of Cysteine and Fe-S Cluster Biosynthesis

Cysteine and its intermediates are essential for survival of virtually all living organisms from bacteria to higher eukaryotes (see reference 233). Cysteine have various important function, in *E. histolytica*, *G. intestinalis*, and *T. vaginalis* and is the major thiol in *E. histolytica* (92) and *G. intestinalis* (36, 108, 109), where it is present in a reduced form. Thus, cysteine plays an important role in maintaining the redox balance of thiols in these organisms. In addition, cysteine provides an inorganic sulfur atom for the biosynthesis of Fe-S clusters, which are important in various proteins, including PFOR, ferredoxin, and hydrogenase. Fe-S clusters have various important functions, including oxidative phosphorylation, electron transfer, and regulation of gene expression and of enzyme activities including substrate binding and activation, sulfur donation, and iron storage (27, 152).

## Heterogeneity of Fe-S Cluster Biosynthesis

Three independent systems are known for the biosynthesis of Fe-S clusters: the ISC, sulfur utilization factor (SUF), and NIF systems (15). While the ISC machinery has a ubiquitous housekeeping function in most organisms, the SUF machinery is involved in the stress response under iron-deficient and oxidative stress conditions in a range of organisms from archaebacteria to certain protists (223, 242, 302). In contrast to the ISC and SUF systems, the NIF machinery is present in only a limited number of organisms, especially anaerobic or microaerophilic bacteria such as nitrogen-fixing bacteria and nondiazotropic *Epsilonprotobacteria*, including *Campylobacter jejuni*, *Helicobacter pylori* (240), and *E. histolytica* (15, 315). There is no precedent for the NIF system in any other eukaryotes.

In contrast to *E. histolytica*, which possesses only the NIF system and lacks both the ISC and SUF systems, *T. vaginalis* and *G. intestinalis* (69, 294, 295, 304), together with *Cryptosporidium parvum* (174), exclusively contain an ISC system, while *Plasmodium falciparum* has both the ISC and SUF systems (83, 284, 331). While the NIF system in *E. histolytica* is localized mainly, if not exclusively, in the cytoplasm (V. Ali and T. Nozaki, unpublished data), the ISC system is localized in the hydrogenosomes and the mitosomes in *T. vaginalis* and *G. intestinalis*, respectively (69, 294, 304), similar to the mitochondrial localization of the ISC system in aerobic mitochondrion-containing eukaryotes. The major Fe-S cluster-containing proteins, ferredoxins and PFOR, play an important role in energy metabolism, electron transfer, and redox regulation and participate in the activation of chemotherapeutics, including metronidazole, in these three "amitochondriate" protists, as described above. Therefore, differences in the biochemical properties and intracellular localization of Fe-S biosynthesis between the "amitochondriate" protists should strongly influence strategies for future drug development.

## Unique Aspects of Sulfur-Containing-Amino-Acid Metabolism

Sulfur-containing-amino-acid metabolism in *E. histolytica* and that in *T. vaginalis* have many striking similarities including (i) the presence of a sulfur-assimilatory de novo cysteine biosynthetic pathway (233, 235, 326); (ii) the presence of a unique enzyme, methionine γ-lyase (MGL), for degradation of sulfur-containing amino acids (184, 202, 301); and (iii) the presence of both phosphorylated and nonphosphorylated serine meta-

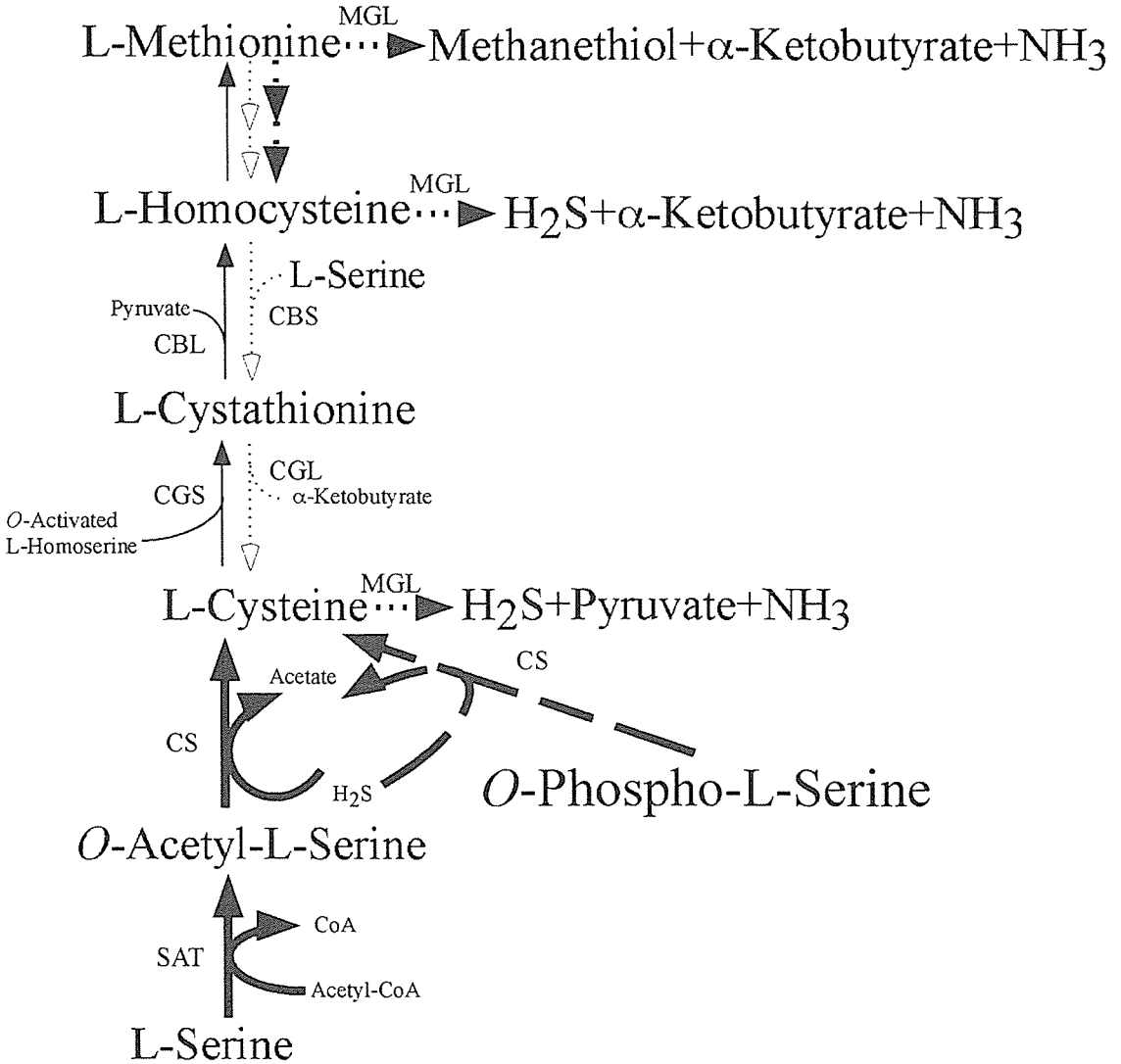


FIG. 3. General scheme of transsulfuration, cysteine biosynthesis, and sulfur amino acid degradation. The schematic diagram shows all pathways present in bacteria. Open arrows with thin dotted lines depict pathways present in mammals; arrows with thick dotted lines represent pathways present in both *E. histolytica* and *T. vaginalis*; and arrows with thick unbroken lines or thick broken lines represent pathways present only in *E. histolytica* or *T. vaginalis*, respectively. CBS, cystathionine β-synthase; CBL, cystathionine β-lyase.

bolic pathways upstream of cysteine metabolism (12–14, 233, 326). Since MGL and two enzymes involved in the cysteine biosynthetic pathway, serine *O*-acetyltransferase and cysteine synthase (CS), are absent in mammals, they are potentially ideal targets for new chemotherapeutic agents against these protozoan parasites. Methionine and cysteine metabolism in protozoan parasites was recently reviewed in detail (233), where we described genome-wide comparisons of pathways and individual enzymes involved in the pathways in representative parasitic protozoa that infect humans. Here, we highlight only two aspects of unique amino acid metabolism: degradation of sulfur-containing amino acids and cysteine biosynthesis.

### Degradation of Sulfur-Containing Amino Acids

MGL (EC 4.4.1.11), which is present in both *E. histolytica* and *T. vaginalis*, catalyzes the decomposition of methionine, homocysteine, cysteine, and some other substituted serine or homoserine analogues, including *O*-acetylserine and *O*-acetyl-L-homoserine, by α- and β-elimination or by α- and γ-elimination (Fig. 3). MGL belongs to the α family of pyridoxal 5′-phosphate (PLP)-dependent enzymes and requires PLP as a cofactor for its activity (206). Both *E. histolytica* and *T. vaginalis* possess two isotypes of MGL which differ in substrate specificities, overall charge (i.e., pI), and other enzymological properties (202, 301). The biochemical properties of MGLs from *Pseudomonas putida* (147), an *Aeromonas* sp. (224), and *Clostridium sporogenes* (165) were also characterized. The MGLs from *E. histolytica* and *T. vaginalis* lack activity toward cystathionine (184, 301), whereas the *P. putida* and *Aeromonas* MGLs utilize cystathionine as a substrate (89). The lack of cystathionine lyase activity in *E. histolytica* and *T. vaginalis* MGLs seems reasonable, since these protozoa lack both forward and reverse transsulfuration pathways (Fig. 3) and thus

do not produce cystathionine as an intermediate (202). The biological significance of MGL includes the production of propionic acid for energy metabolism (18) and the degradation of toxic sulfur-containing amino acids (233). Sulfur-containing amino acids, particularly homocysteine, are implicated in cardiovascular diseases, Alzheimer's disease, and dementia (305).

Specific functions for each MGL isotype and the significance of the apparent redundancy of the two isotypes are not understood for either *E. histolytica* or *T. vaginalis*. Our recent DNA microarray analysis showed that mRNA expression of MGL1 and that of MGL2 were comparable (unpublished data), supporting the previous observation that the two MGL isotypes were expressed in comparable amounts (301). Interestingly, the expression of MGL1, but not that of MGL2, was upregulated >14-fold at 1 day after the amoebae were inoculated into the mouse intestine (105), suggesting an in vivo role for this MGL isotype for adaptation to the intestinal environment or, alternatively, MGL1 overexpression being part of the stress response.

MGL activity was not detected in crude extracts from the other anaerobic protozoan parasites (e.g., the related reptilian species *E. invadens*, *T. foetus*, *Trichomitus batrachorum*, and *G. intestinalis*) (184, 299), suggesting that the presence of MGL is not directly associated with anaerobic metabolism. Since MGL was also found in the closely related but nonpathogenic *Entamoeba* species *E. dispar*, MGL is unlikely to be involved in the virulence mechanism but rather is likely to be associated with commensal adaptation. Phylogenetic analysis suggested that MGL isotypes of *E. histolytica* or *T. vaginalis* were most likely obtained from an ancestral archaeal or bacterial organism, respectively, by lateral gene transfer (301).

### Structural Differences between Protozoan MGL and Mammalian CGL

A number of PLP-dependent enzymes are involved in the metabolism of cysteine, homocysteine, and methionine. These enzymes include cystathionine γ-lyase (CGL), cystathionine γ-synthase (CGS), cystathionine β-lyase, MGL, and CS. Mammals lack the forward transsulfuration pathway (cysteine to methionine) and possess only the reverse transsulfuration pathway (methionine to cysteine) (233). One should be cautious that drugs targeting MGL are designed in such a way that they do not affect mammalian CGL. Recently, a crystal structure of yeast CGL, which has >50% amino acid identity to human CGL, has been resolved (210). We and other groups have also determined the tertiary structures of MGL and CGL by X-ray crystallography (unpublished data). Such structural comparisons as well as amino acid alignments (210) revealed conserved amino acids that participate in the catalytic activity of γ-elimination. MGLs from *E. histolytica* (301), *T. vaginalis* (202) (PDB accession no. 1E5F), and *P. putida* (214); CGL from yeast (210); and CGS from *E. coli* (50) are homotetramers, and their overall crystal structures are similar. The tetramer consists of two active dimers (subunits A/C and B/D) related by a twofold axis. While both CGL and MGL catalyze γ-elimination reactions, they have differential substrate preferences. Methionine, which contains a hydrophobic end group, is the preferred substrate of MGL, whereas cystathionine, which has a peptide group instead of a methyl moiety, is the preferred substrate of CGL. The overlay of the active sites of yeast CGL (210) and *T. vaginalis* MGL revealed structural explanations for the substrate preference. The conserved amino acids of *E. histolytica* MGL1 (Phe-44, Met-84, Cys-110, and Val-331) were present in all known MGLs and generally missing in other PLP-dependent enzymes (301). The conserved Glu-333 of yeast CGL, which binds to cystathionine, is replaced with Val-331 in all known MGLs (301). Similarly, the conserved Phe-44 is absent in CGL, and Cys-110 is replaced by glycine, which affects the substrate specificity (210). The phenyl group of Phe-44 of MGL forms a hydrophobic contact area together with the side chains of Leu-55 and Ile-59 to bind the methyl group of methionine. Structural comparisons also provide an explanation for the remarkable differences in cystathionine utilization between MGL and cystathionine β-lyase, CGS, and CGL (210). The three latter enzymes have a conserved glutamate (E333 in yeast CGL) which binds to the peptide group of cystathionine or the amino group of cysteine. The active sites of CGLs from yeast or human are virtually identical to that of *E. coli* CGS. Both CGL and bacterial CGS have γ-synthase and γ-lyase activities, depending on their position in metabolic pathways and the available substrate. Remarkable differences in the structure of the active site of protozoan MGL compared with that of mammalian CGL reinforce the premise that substrate analogues that specifically target parasite MGL, but not human CGL, can be designed. It should also be noted, however, that many important residues, including the ones involved in PLP binding, are shared by protozoan MGL and the related enzymes from human and bacteria. For instance, hydrophobic residues (Ile-55 and Leu-59) in the active site are shared by both yeast and human CGLs, similar to the case for MGLs. A helical structure located at the amino terminus of MGL was shown to cover the cysteine-binding pocket in *E. coli* CGS and CGL in a manner similar to that found in MGL (210). The crystal structure studies on *E. histolytica* MGL isotypes currently being conducted should give insights into detailed structural differences between amoebic MGL and human CGL and between the two MGL isotypes, which could lead to the design of new inhibitors and prodrugs.

### Drug Development Targeting Methionine γ-Lyase

As explained above, MGL represents an ideal target for the development of new drugs against *E. histolytica* and *T. vaginalis*. A fluorinated derivative of methionine, L-trifluoromethionine or L-*S*-(trifluoromethyl)homocysteine (TFM), was reported to be a microbial growth inhibitor 40 years ago (342). TFM has good activity against a variety of bacteria, including *Porphyromonas gingivalis* (338), *Clostridium*, and *Bacteroides* (96). TFM is decomposed into α-ketobutyrate, ammonia, and trifluoromethanethiol ($CF_3SH$) by MGL. The last compound is unstable under physiological conditions and nonenzymatically breaks down to carbonothionic difluoride ($CSF_2$), which is a potent cross-linker of primary amine groups (16, 262). Thus, TFM or its analogues are highly effective against many anaerobic bacteria that cause botulism (*Clostridium botulinum*), colitis (*Clostridium difficile*), tooth decay (*Porphyromonas* species), and intra-abdominal infections (*Bacteroides* species) (96). Complete inhibition of growth occurred at 1 mM TFM in

*P. gingivalis*. TFM also showed significant in vivo efficacy against peritoneal *P. gingivalis* infection in mice (338).

TFM was recently shown to be effective against *T. vaginalis* (53) and *E. histolytica* in vitro (301). TFM effectively killed *T. vaginalis* trophozoites within 24 h at 5 μg/ml in an in vitro culture. TFM also killed *E. histolytica* trophozoites after 48 h with an $IC_{50}$ of 18 μM in an axenic culture. In addition, peritoneal administration of TFM (40 mg/kg of body weight) cured peritoneal infection by *T. vaginalis* in mice within 24 h (53). Five of six mice treated with TFM at 40 mg/kg had no lesions, while a lower dose of 12 mg/kg resulted in a cure rate of 70% (53). TFM also successfully cured experimental amoebic infection. Either intraperitoneal or subcutaneous administration of TFM (36 mg/kg of body weight) successfully cured liver abscesses in hamsters, without notable side effects (unpublished data). In addition, intraperitoneal administration of TFM also partially cured intestinal amoebiasis in a C3H/HeJ mouse model (139) of infection with a direct inoculation of trophozoites into the cecum (unpublished data). The major side effect in the treated mice was weight loss, suggesting that there may be species-dependent side effects with TFM. We are currently synthesizing and testing a variety of TFM derivatives to increase the efficacy as well as to decrease the side effects. We have found amide derivatives of TFM that are highly effective against in vitro cultures of *E. histolytica* within 24 to 48 h, with an $IC_{50}$ of 1.5 to 2 μM. These derivatives are likely lead compounds for the development of new drugs against *E. histolytica* and *T. vaginalis* (unpublished data).

A compound antagonistic to MGL, propargylglycine, was not deleterious to amoebic trophozoites in axenic cultures (301). Propargylglycine at 20 μM did not affect *E. histolytica* growth, although it did completely inhibit MGL activity after 24 h. In addition, propargylglycine did not kill the amoebae in polyxenic cultures (unpublished data). These data indicate that MGL is not essential for *E. histolytica* survival and growth under these in vitro conditions. However, this does not necessarily rule out the possibility that MGL may be involved in the in vivo survival and virulence of these pathogens in mammalian hosts. In contrast, *G. intestinalis* possesses a rather distinct metabolism of sulfur-containing amino acids and does not contain MGL. TFM showed no effect on the growth of *G. intestinalis*, which is consistent with the absence of MGL in *G. intestinalis* (53).

### Sulfur-Assimilatory De Novo Cysteine Biosynthesis

The cysteine biosynthetic pathway plays an important role in the incorporation of inorganic sulfur into organic compounds in bacteria, plants, and parasitic protists, including *E. histolytica*, *T. vaginalis*, and *Trypanosoma cruzi*, which is the etiological agent of Chagas' disease (American trypanosomiasis) (233–235, 237, 238, 326). The pathway has been extensively studied in bacteria and plants (126, 239, 270). In plants, the pathway is differentially localized in at least three compartments: the cytosol, mitochondria, and chloroplasts (127, 128, 130, 230, 270, 272, 332). In *E. histolytica*, where neither conventional mitochondria nor chloroplasts are present, the pathway exists exclusively in the cytosol. The pathway is also likely present in the cytosol in *T. vaginalis* and *T. cruzi*, because neither of the two enzymes in the pathway contains organelle-targeting sequences.

In bacteria and plants, which can reduce incorporated sulfate to sulfide via sulfite, extracellular sulfate is first imported by sulfate transporters (270–272). After sulfate activation and reduction, incorporated sulfate (+6) is derivatized to sulfide (−2), which serves as an acceptor for the alanyl moiety of the donor molecule. Serine *O*-acetyltransferase (SAT) (EC 2.3.1.30) catalyzes the formation of the alanyl donor *O*-acetylserine from serine and acetyl-CoA (235) (Fig. 3). CS [*O*-acetyl-L-serine (thiol)-lyase] (EC 4.2.99.8) then catalyzes the production of L-cysteine by the transfer of the alanyl moiety of *O*-acetylserine to sulfide (234, 238). Since *E. histolytica* and *T. vaginalis* apparently lack a sulfate reduction pathway, they likely utilize sulfide derived from the iron-sulfur proteins from ingested bacteria in the intestine or vagina, respectively. In contrast to these parasites, animals lack the sulfur-assimilatory pathway and thus require exogenous methionine as a sulfur source.

Although it was previously shown that *E. histolytica* possesses one SAT and two allelic isotypes of CS (234, 235), our recent survey of CS and SAT in the *E. histolytica* genome database (185) revealed a third CS isotype and two additional SAT isotypes (unpublished data). There are several unique features of the amoebic SAT and CS which are remarkably different from those of other organisms. It has been shown that SAT1 (previously named SAT) is a regulated key enzyme of the cysteine biosynthetic pathway in *E. histolytica* (235). SAT1 is regulated by allosteric negative feedback by cysteine and cystine. Negative regulation by L-cystine has no precedent, highlighting the amoebic SAT1 as a unique enzyme. Another unique aspect of the amoebic SAT1 is a lack of protein-protein interaction with CS. In both bacteria and plants, CS and SAT form a heteromeric complex with a molecular mass of several hundred kilodaltons (70, 71, 164). However, both CS1 (and CS2) and SAT1 form a homodimer, and they do not interact with each other under physiological conditions. The lack of CS-SAT interaction was unequivocally demonstrated by three biochemical and genetic methods: (i) separation by conventional chromatography during purification from the crude cell lysate, (ii) an inability to coimmunoaffinity purify the proteins, and (iii) the yeast two-hybrid system (235). The second and third SATs (SAT2 and SAT3, respectively) showed 73% and 48% amino acid identity to SAT1, respectively. *E. histolytica* SAT3 possesses a 25- to 30-amino-acid extension at the carboxyl terminus and a low isoelectric point (5.7) compared to *E. histolytica* SAT1 and *E. histolytica* SAT2. These features may favor an interaction with *E. histolytica* CS, in particular with *E. histolytica* CS3, which possesses the highest pI (8.17) among the three isotypes (unpublished data).

Among the three CS isotypes (CS1 to -3), two CS proteins (CS1 and CS2) are identical except for two conservative amino acid changes (234). CS3 is divergent from the other two isotypes, with ~83% amino acid identity to CS1 and CS2 (unpublished data). While both CS1 and CS2 are localized in the cytoplasm of *E. histolytica*, similar to prokaryotic CysK and CysM, the intracellular distribution of CS3 remains unknown. In plants, three isotypes, i.e., CS-A, CS-B, and CS-C, are localized to the cytoplasm, chloroplasts, and mitochondria, respectively (130, 230, 270–273). CS1, CS2, and CS3 rescued the

growth defect of a CysK-deficient *E. coli* strain, suggesting that all these isoforms of CS are functional as CysK in a heterologous organism (234; unpublished data). *E. dispar* also possesses two CS isotypes, CS1 and CS2 (82 to 83% mutual identity) (238). *E. dispar* CS1 and CS2 correspond to *E. histolytica* CS1/2 and 3, respectively. Taken together, this information indicates that *Entamoeba* possesses at least two classes of CS isotypes, each with a distinct pI. All of these CS isotypes lack signal sequences or organelle-targeting sequences, suggesting a cytosolic location. The presence of multiple cytosolic CS isotypes in the nonpathogenic *E. dispar* species suggests that this enzyme is not directly associated with the pathogenicity of the amoeba but that it plays an important housekeeping role in *Entamoeba*.

Recently, sulfur-assimilatory cysteine biosynthesis was characterized in *T. vaginalis* (326). The genomic analysis of *T. vaginalis* revealed six copies of CS but no SAT. Enzymological characterization of CS indicates that *T. vaginalis* CS is able to utilize *O*-phosphoserine as well as *O*-acetylserine as an alanyl donor. Although *T. vaginalis* lacks SAT and is thus unable to produce *O*-acetylserine, *T. vaginalis* also possesses three copies each of 3-phosphoglycerate dehydrogenase and phosphoserine aminotransferase and presumably is able to form *O*-phosphoserine from 3-phosphoglycerate derived from glycolysis.

The biological significance of cysteine synthesis in "amitochondriate" protists is still not yet fully understood. It was shown that cysteine is a major intracellular thiol in these organisms (36, 92, 106, 107, 110). L-Cysteine could not be replaced by any other thiols or reducing agents in the growth medium (109), suggesting that cysteine is essential for growth and survival of *E. histolytica* and *G. intestinalis* (36, 108, 109). In addition, cysteine also plays a role in the antioxidative defense of *E. histolytica* (235). Overexpression of CS (2- to 3-fold) but not of SAT (13-fold) by episomal introduction of multicopy plasmids resulted in only a 2-fold increase in the intracellular thiol content and the hydrogen peroxide resistance (235). These data indicate that the intracellular concentration of CS1 (or CS2) but not SAT1 mainly affects the thiol content. One of the major unsolved questions related to the pathway is why these protists possess apparently redundant systems, while they have lost many other metabolic pathways by reductive evolution.

### Cysteine Synthase as a Drug Target

CS from *E. histolytica* utilizes a variety of alanyl acceptors, including some thiols or N-heterocyclic compounds besides sulfide (234), similar to the case for plant CS (144). The specificity of these alanyl acceptors varies between the organisms from which the CS is derived. The spectrum of the alanyl acceptors for CS was most extensively studied for the plant enzymes. For instance, spinach CS utilizes a wide range of alanyl acceptors to form the corresponding β-substituted alanines. These products include L-quisqualic acid, L-mimosine, L-willardiine, L-isowillardiine, β-(pyrazol-1-yl)-L-alanine, β-(1,2,4-triazol-1-yl)-L-alanine, and β-(3-amino-1,2,4-triazol-1-yl)-L-alanine (144, 145, 201). Some of these amino acids are toxic to or physiologically active in organisms in which they do not normally occur. For example, L-quisqualic acid, present in *Quisqualis* spp., is a neuroexcitory amino acid and is utilized as a vermicide in Chinese medicine. L-Mimosine is present in *Mimosa* and *Leucaena* spp. and is a thyrotoxic amino acid that causes loss of hair in growing animals. β-(3-Isoxazolin-5-on-2-yl)-L-alanine from *Pisum* and *Lathyrus* spp. showed antimitotic activity in *Saccharomyces cerevisiae*. Commonly used herbicides (1,2,4-triazole and 3-hydroxy-5-methylisoxazole) are alanylated by CS (146).

*E. histolytica* CS also uses sulfide, 1,2,4-triazol, isoxazolin-5-one, pyrazole, and cyanide as alanyl acceptors. The specific activity against these acceptors is, however, 9- to 4,000-fold less than that for sulfide (234). As seen for triazole, pyrazole, tetrazole, and their derivatives, which have been used as herbicides targeting plant CS, these compounds showed toxic effects on *E. histolytica* in vitro (unpublished data), necessitating a further screening of CS substrates with amoebacidal activity. Thiol derivatives of tetrazole and triazole are highly cytotoxic against *E. histolytica* cultures in vitro (unpublished data). Recently, it was also demonstrated that reduced forms of pyrazole, pyrazolines, and their derivatives are highly effective against amoebiasis (3–5, 41). The mode of action and toxicity of these derivatives against mammalian cells have not been studied. Similarly, 1,2,4-triazine and triazine derivatives were also cytotoxic to African trypanosomes and were selective between host cells and parasites (24, 66). Triazine-substituted polyamines have also been viewed as excellent chemotherapeutics against trypanosomiasis based on an in vitro study (160). The triazine derivatives showed amoebacidal activity in vitro, with $IC_{50}$s lower than that of metronidazole (287). However, neither their target in *E. histolytica* trophozoites nor their mode of action has been elucidated. None of these compounds has been tested in vivo. It is conceivable that some of these derivatives target sulfur-containing-amino-acid metabolism and in particular CS or MGL.

## CONCLUSIONS

Due to an immediate necessity for new drugs against parasitic infections, parasite-specific targets have been exploited. Sulfur-containing-amino-acid metabolism represents one promising source of such targets. The exclusive existence of sulfur-assimilatory de novo cysteine biosynthesis and the unique enzyme for degradation of sulfur-containing amino acids in *E. histolytica* and *T. vaginalis* verified that these pathways are ideal targets for drug development. Sulfur-containing-amino-acid metabolism and its intermediates play various pivotal roles in parasitic protozoa. Two proteins, MGL and CS, have most often been exploited to develop antiprotozoan drugs. Currently, L-S-(trifluoromethyl)homocysteine and its derivatives are being extensively synthesized and tested for in vitro and in vivo efficacy against liver and intestinal amoebiasis in animals. Once the tertiary structures of the substrate-binding pockets of MGL and mammalian CGL are determined, the rational design of compounds targeting MGL without adverse effects on the host CGL will become possible.

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Molecular & Biochemical Parasitology 147 (2006) 163–176
MOLECULAR & BIOCHEMICAL PARASITOLOGY

# Impact of intestinal colonization and invasion on the *Entamoeba histolytica* transcriptome

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Received 7 November 2005; received in revised form 7 February 2006; accepted 8 February 2006
Available online 7 March 2006

**Abstract**

A genome-wide transcriptional analysis of *Entamoeba histolytica* was performed on trophozoites isolated from the colon of six infected mice and from in vitro culture. An Affymetrix platform gene expression array was designed for this analysis that included probe sets for 9435 open reading frames (ORFs) and 9066 5′ and 3′ flanking regions. Transcripts were detected for >80% of all ORFs. A total of 523 transcripts (5.2% of all *E. histolytica* genes) were significantly changed in amebae isolated from the intestine on Days 1 and 29 after infection: 326 and 109 solely on Days 1 and 29, and 88 on both days. Quantitative real-time reverse transcriptase PCR confirmed these changes in 11/12 genes tested using mRNA isolated from an additional six mice. Adaptation to the intestinal environment was accompanied by increases in a subset of cell signaling genes including transmembrane kinases, ras and rho family GTPases, and calcium binding proteins. Significant decreases in mRNA abundance for genes involved in glycolysis and concomitant increases in lipases were consistent with a change in energy metabolism. Defense against bacteria present in the intestine (but lacking from in vitro culture) was suggested by alterations in mRNA levels of genes similar to the AIG1 plant antibacterial proteins. Decreases in oxygen detoxification pathways were observed as expected in the anaerobic colonic lumen. Of the known virulence factors the most remarkable changes were a 20–35-fold increase in a cysteine proteinase four-like gene, and a 2–3-fold decrease in two members of the Gal/GalNAc lectin light subunit family. Control of the observed changes in mRNA abundance in the intestine might potentially rest with four related proteins with DNA binding domains that were down-regulated 6–16-fold in the intestinal environment. In conclusion, the first genome-wide analysis of the transcriptome of *E. histolytica* demonstrated that the vast majority of genes are transcribed in trophozoites, and that in the host intestine trophozoites altered the expression of mRNAs for genes implicated in metabolism, oxygen defense, cell signaling, virulence, antibacterial activity, and DNA binding.


*Keywords:* *E. histolytica*; Oligonucleotide array sequence analysis; Gene expression profiling; Entamoeba/parasitology; Protozoan metabolism; Computational biology

## 1. Introduction

The early branching eukaryote *Entamoeba histolytica* is the etiologic agent of amebic dysentery and liver abscess. These diseases result in substantial morbidity and mortality in the developing world. To colonize and invade humans, trophozoites

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doi:10.1016/j.molbiopara.2006.02.007

must adapt to the complex anaerobic environment of the colon. *E. histolytica* is an aerotolerant anaerobe that derives energy in vitro from the glycolytic and fermentation pathways. It has undergone a secondary loss of mitochondria, retaining only a remnant organelle that lacks a mitochondrial genome [1]. Energy is acquired from glycolytic and fermentation pathways that include many laterally transferred genes of bacterial origin [2,3].

The ability of *E. histolytica* to cause disease in animal models requires not only an energy source for the parasite but also virulence factors that mediate contact-dependent killing and phagocytosis of host cells and intestinal invasion. Virulence factors include cysteine proteases, the galactose/*N*-acetyl D-galactosamine-inhibitable (Gal/GalNAc) adherence lectin, and amebapore pore-forming proteins [4,5].

To date our understanding of virulence has been limited by the inability to study parasite gene expression in vivo at a comprehensive genome-wide scale. In order to identify pathways and genes that are involved in intestinal adaptation and invasion, we designed a gene expression array representing 9435 open reading frames (ORFs). This array was used to perform a transcriptional analysis of *E. histolytica* isolated from mouse ceca at early and late time points of infection. The changes in mRNA abundance and their potential impact on the biology of the parasite during intestinal colonization and invasion are discussed.

## 2. Materials and methods

### 2.1. Cultivation of E. histolytica

*E. histolytica* strain HM1:IMSS trophozoites were grown at 37 °C in TYI-S-33 medium containing penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO/BRL) [6]. To cultivate amebae from the intestine, infected mouse cecal contents were added to amebic growth medium and amebae were counted on Day 4.

### 2.2. Mouse model of amebic colitis

CBA/J mice were infected by luminal injection of $2 \times 10^6$ trophozoites into the ceca, exposed by laproscopic surgery [7]. Infected mice were sacrificed at Day 1 or Day 29 after infection.

### 2.3. RNA isolation

Approximately 2.4–5 $\times$ $10^5$ *E. histolytica* trophozoites from culture were lysed by the addition of 0.8 ml of Trizol reagent (Invitrogen), and total RNA isolated by the manufacturer's directions. RNA from infected mice was purified from cecal contents of infected mice immediately after sacrifice. Cecal contents were washed in ice cold PBS and collected by centrifugation for 10 s at 14,000 rpm. One milliliter of Trizol (Invitrogen) was added to the cecal contents and an initial RNA preparation performed according to manufacturer's directions. Total RNA was treated with DNAse I (Roche Applied Science) to remove contaminating genomic DNA. RNA greater than 200 nucleotides in length was separated from total RNA by the RNeasy protocol (Qiagen). Control RNA was isolated from the ceca of mock-infected CBA mice by an identical protocol to test for cross-hybridization of the array with murine transcripts.

### 2.4. Design of Affymetrix custom array

A custom array (E_his-1a520285) was designed using information generated from the *E. histolytica* genome sequencing project release date 12/08/04 [3]. The ORF probe sets were preferentially selected from the 600 bases proximal to the 3′ end of the *E. histolytica* sequences. Probe sets consisted of 8–16 25mer oligonucleotide probes. Probe pairs consisted of a perfect match to the available sequence, and a control probe, which contained a mismatch at position 13. Probe sets were generated to 7712 sequences that represented 9435 ORFs, and consisted of both (_at) probe sets that represent single gene and (_x_at) probe sets. The latter may cross-hybridize in an unpredictable manner with sequences other than the main target. Some sequences were represented by both (_at) and (_x_at) probe sets, therefore the total number of probe sets for ORF's was 11,397. As 1723 predicted genes in the *E. histolytica* genome were identical to other ORF's on the chip, or were so highly similar in sequence as to make it impossible to design a unique probe set, these genes were represented by a probe set with the suffix (_s) and the additional represented genes are listed in Supplemental Table 1. Probes to highly repetitive sequences (LINE and SINE elements, rRNA, and tRNA genes) were not included in the array. *E. histolytica* sequences similar to *E. coli*, mouse or human sequences were also removed. This "pruning" process deleted approximately 5% (505) of the *E. histolytica* ORFs from the designed array.

Probe sets spotted on the Affymetrix array represented both ORF and selected UTR regions. The UTR probes were not used in the analysis described in this paper but are included in the MIAME-compliant data sets (supplemental data) [8]. In the design of the UTR probes 1–15 probe pairs were chosen with a desired gap between probes of 20–70 bp from available sequence information. In cases where the UTR sequence was >1000 bp in length, probe sets were chosen from the first and last 500 bases. These sets were distinguished by -a and -b suffixes. There are 12,777 UTR probe sets, which represented 9066 5′ and 3′ UTR's. Seven hundred and forty-one intergenic sequences were not represented on these arrays due to lack of space on the array.

### 2.5. Affymetrix DNA chip hybridization and analysis

Isolated total RNA was checked for integrity and concentration using the Agilent Bioanalyzer 2100 RNA nanochip Assay (Agilent Technologies). Fifty nanograms of total RNA was converted into cDNA using the SPIA™ isothermal linear RNA amplification system, which employs a single chimeric primer, DNA polymerase with strand displacement activity and RNase H (NuGen Technologies). RNase H cleaves the RNA portion of the heteroduplex at one end of the double-stranded cDNA, thus generating a unique partial duplex cDNA with a single-stranded DNA tail at the 3′ end of the second strand cDNA.

This tail is the priming site for the SPIA(tm) amplification step. The sequence of the SPIA(tm) amplification primer, a chimeric DNA/RNA primer, is complementary to the sequence of the single-stranded 3′ end of the second strand cDNA in the partial duplex, which is extended along the template DNA by DNA polymerase. Strand displacement DNA synthesis results in displacement of the prior primer extension product away from the template DNA. This cycle of primer binding, extension, displacement and cleavage results in the generation of the amplified product.

The resulting ~5 μg of biotinylated cDNA was hybridized to the E_his-1a520285 custom array (Affymetrix). In cases where the proportion of amebic RNA was less than 50% of total RNA (as determined by the ratio of amebic to mouse rRNA), and when labeling RNA derived from the sham infected CBA mouse (as a control), two separate labeling reactions were performed and pooled for the hybridization. The arrays were washed, stained with streptavidin-phycoerythrin (Molecular Probes), following the standard Affymetrix protocol for eukaryotic targets (Affymetrix). The arrays were scanned with an Affymetrix Gene Chip scanner 3000l and report files were generated to determine the percentage of present calls of each array.

In experiments using amebic mRNA isolated from the cecum of mice, only microarray hybridizations with low background hybridization values and raw probe set intensities with an average signal of greater or equal to 180 were included in the analyses (see Supplemental Table 2). To test for cross-hybridization with murine transcripts, the arrays were hybridized to mouse RNA and to cecal contents from mock-infected CBA mice.

Raw data from the arrays was normalized at probe level using gcRMA algorithm [9]. The detection calls (present, marginal, absent) for each probe set was obtained using the GCOS system (http://www.affymetrix.com/products/software/specific/gcos.affx). Only genes with at least one present call across all the compared hybridizations were kept for downstream statistical analyses. Significance of gene expression was determined using the significance analysis of microarrays (SAM) statistical program (http://www-stat.stanford.edu/~tibs/SAM/). Genes significantly modulated in vivo in the intestine were defined by the following criteria: a calculated $q$-value of less than 0.05 from SAM analysis; probe sets with a normalized signal intensity greater than 50; and changes greater than two-fold compared to cultured amebae. The normalized intensity value for each probe set, probe set ID, gene ID, GenBank #, fold difference between conditions and statistical significance from SAM analysis are shown in Supplemental Table 4. Additional GO terms and pfam domains were provided in each list of the identified differentially expressed genes and the data is shown in Supplemental Table 5. The whole dataset was deposited at VBIExpress (http://vbiexpress.vbi.vt.edu/VbiExpress/public), which follows MIAME guidelines.

### 2.6. qRT-PCR

Reverse transcription real-time PCR (qRT-PCR) was used to independently measure mRNA abundance. The Superscript II enzyme (Invitrogen) was used according to the manufacturer's directions, with qRT-PCR analysis of the amebic transcripts performed in an Opticon II (MJ Research) machine. The reverse transcription of both sense and antisense RNA was performed using random sequence hexamers. The cDNA was subjected to 40 amplification cycles with HotStar Taq (Qiagen) Opticon. Primers were designed to amplify 100–300 base pairs using the *E. histolytica* Genome Sequencing Project and the Primer3 program (Supplemental Table 4) [3]. The fluorescent dye SYBR Green I (Molecular Probes) was used to detect the amplified cDNA. Continuous SYBR Green I monitoring during amplification using the MJR Opticon II machine was done according to the manufacturer's recommendations. All real-time amplification reactions were performed in duplicate and the resulting fluorescent values averaged. In all experiments utilizing qRT-PCR the cycle threshold values ($C_T$, the cycle number at which fluorescence exceeds the threshold value) were linked to the quantity of initial DNA after calibration of the amplification efficiency of the primer pair utilized [10]. To identify the most stably expressed transcripts to determine the quantity of amebic RNA isolated from the mouse ceca, the geNORM program of Vandesompele et al. [11] was used to compare a data set of "housekeeping" genes. This allowed us to empirically determine the most stably expressed of the *E. histolytica* transcripts (RNA pol II L, TSA, and cysteine protease 19). The geographic mean of the levels of these transcripts was used to compensate for the variation in the amount of amebic mRNA isolated from the infected mouse. Statistical significance was determined using either the Student's $t$-test or Welch's approximate $t$-test, which does not assume equal variance of in the two populations (InStat 2.03 program (GraphPad Software)).

## 3. Results and discussion

### 3.1. Amebic burden in infected mice

Chronic non-healing amebic colitis in the cecum of C3H and CBA mice occurs after intracecal injection of *E. histolytica* trophozoites [7]. We measured the number of amebae residing in the cecal lumen at 1, 4, 9 and 29 days after infection by determining the level of the RNA polymerase II L protein mRNA by qRT-PCR (Fig. 1A), and by semi-quantitative amebic culture (Fig. 1B). The *E. histolytica* transcriptome was measured at Days 1 and 29 after infection for two reasons, first that the amebae were most abundant then, and because these represented early and late time points in the adaptation to intestinal growth.

### 3.2. E. histolytica intestinal transcriptome

To identify changes in gene transcription that occurred in vivo, amebic RNA was isolated from the cecal lumen of three mice early in infection (Day 1) and three mice late in infection (Day 29), and from the cultured parasites used to initially infect the mice. Transcripts were detected for more than 80% of the ORF probes sets on the array with cDNA generated from in vitro cultivated amebae (Supplemental Table 5). A total of 523 transcripts (5.2% of all *E. histolytica* genes) were signif-

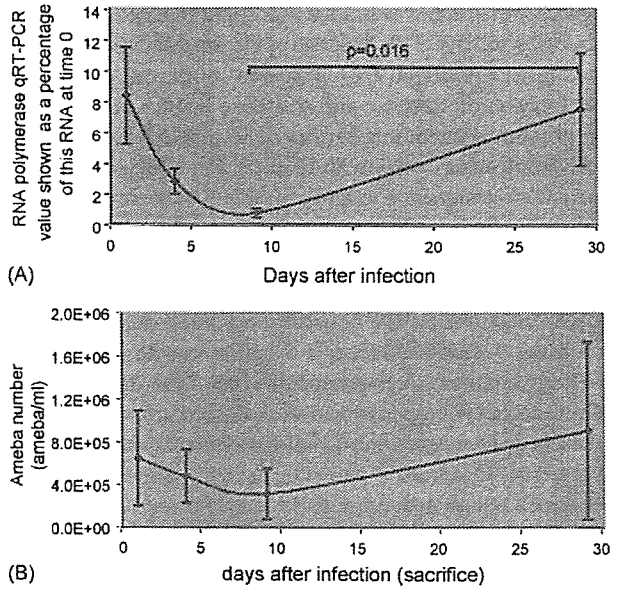


Fig. 1. Burden of *E. histolytica* in the Intestine: (A) cecal contents were washed in ice-cold PBS, and collected by centrifugation mRNA levels were measured by qRT-PCR of a relatively invariant mRNA RNA polymerase II L. *y*-axis shows RNA polymerase II L transcript levels relative to the RNA polymerase II L mRNA in the caecum right after injection (100%) and (B) semi-quantitative culture measurements of the abundance of *E. histolytica* in the cecum at Days 1–29 after infection by intracecal injection of trophozoites.

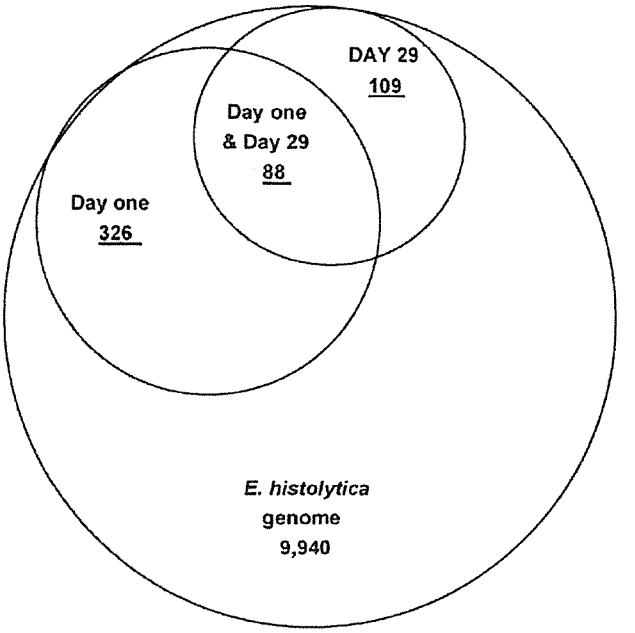


Fig. 2. Venn diagram of differentially expressed transcripts at 1 and 29 days after infection. Transcripts were included on the basis of a ≥two-fold difference and a *q*-value of ≤0.05. Some transcripts, however, while statistically changed at only Day 1 or 29, were still over two-fold different at the other day although no longer statistically significant (see Supplemental Table 5).

icantly modulated in vivo in the intestine as defined by the following criteria: probe sets with a normalized average signal intensity greater than 50 in at least one of the compared conditions; changes greater than two-fold compared to cultured amebae; and a calculated *q*-value of less than 0.05 from SAM analysis. Compared to cultured amebae, 326 of these transcripts were significantly changed only at Day 1, 109 were significantly changed only at Day 29, and 88 were changed at both time points (Fig. 2). Approximately 45% of these genes were annotated as hypothetical or of unknown function. The modulated genes were grouped into broad categories based on the genome annotation (Fig. 3) and discussed below.

### 3.3. Verification of the microarray data

To validate the changes in mRNA abundance detected by microarray analysis, RNA was isolated from the ceca of an additional three mice from Day 1 to Day 29. qRT-PCR was performed on six differentially expressed genes and five invariant genes, based on Affymetrix analysis. qRT-PCR data agreed with the microarray data for 10/11 transcripts, and was statistically significant in 9/11 of the genes tested (Table 1). The only transcript that gave inconsistent expression data between qRT-PCR and microarrays encoded cysteine protease 9 (CP9). The cysteine proteases are part of a large gene family and, while probes were carefully chosen, it is possible that cross-hybridization with the other highly modulated CP transcripts may account for this result. Overall we concluded that there was an excellent correlation between the array data and the qRT-PCR results.

### 3.4. Day 1 versus Day 29 adaptation to the intestine

At Day 1 there was a general down-regulation of genes involved in metabolism and protein translation. The mRNA levels of many of these genes returned to levels observed in cultured amebae by Day 29. We considered if this could be a reflection of an initial stress response on Day 1 to the gut environment. In fact two known stress-related transcripts (an Hsp70 and an Hsp90 gene) had significantly increased expression at Day 1. However, transcripts from two other members of the Hsp70 heat shock protein family, as well as two different transcripts encoding DNAJ proteins, and a RAD52 mRNA, were decreased in abundance at Day 1. Interestingly none of the transcripts found to be modulated in response to in vitro heat shock by Bruchhaus et al. were significantly changed in vivo at either Day 1 or Day 29 [12]. We concluded that changes in gene expression at Day 1 but not Day 29 were likely to be at least in part due to the initial stress of intestinal adaptation on the first day after infection.

### 3.5. Cysteine proteinase gene expression

Cysteine proteinases are hypothesized to facilitate invasion by degrading the gut extracellular matrix and inhibition of the their expression with antisense RNA or with chemical inhibitors blocks in vivo virulence. Twenty of the 29 cysteine proteinase genes were expressed above background, and of these 12 had been previously described [12]. The expression of 11 of 12 previously identified genes was consistent with prior studies (Table 2). The CP1 gene that is unique to *E. histolytica* (absent in the closely related but nonpathogen *E. dispar*) increased two-